TISSUE MICROARRAYS IN DRUG DISCOVERY

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Advances in molecular methods have massively facilitated the discovery of potential molecular targets for gene-specific therapy. Accelerated lead discovery has at the same time generated a massive demand for thorough validation of such putative targets. Very often human tissue analysis is needed for this purpose. However, the need to analyse large numbers of well-characterized human tissues constitutes a major bottleneck in drug discovery and development. Traditional tissue analysis in a slide-by-slide manner is slow, expensive and difficult to standardize. In addition, precious specimens, such as tissue samples from clinical studies, are usually exhausted after a few analyses. The tissue microarray technology overcomes these shortcomings as it allows the simultaneous analysis of up to 1,000 minute tissue samples in a single experiment. This article will review how high-throughput tissue microarray analyses can dramatically facilitate translational research at several different levels.

MICROTOME
Device for cutting histological sections from tissue blocks.

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High-throughput molecular technologies for expression screening, compound screening or functional analyses have enabled the identification of numerous putative target genes for new disease therapies, including anticancer agents. The need to analyse large numbers of well-characterized human tissues constitutes a major bottleneck for the further evaluation of such leads. Large-scale human tissue analysis is crucial in many phases of drug discovery and development. This is particularly true for cancer research: the variety of aberrant pathways that can lead to morphologically identical tumours means that a large number of tumours must be analysed in cancer studies to obtain a full representation of all genetic subtypes of a tumour type of interest. In cancer studies, significant human tissue analyses are needed at three different phases of drug development. First, information on the prevalence of target gene alterations in one or several cancer types is needed to assess the potential market size of a new therapeutic target. Second, predictive methods must be developed to identify those patients with the highest likelihood of benefiting from a new therapy. And last, once a clinical trial has been initiated, it is crucial to preserve tissues from study patients for subsequent molecular analyses to be able to retrospectively identify predictive molecular markers. Previous methods for tissue analyses were either based on homogenized tissue samples, which does not allow the allocation of molecular findings to individual cell types, or on the analysis of conventional tissue sections, which is a slow and tissue-consuming effort. Tissue microarrays (TMAs) significantly facilitate and accelerate *in situ* analysis of tissues in target discovery and validation (FIG. 1). Although the TMA technique has mostly been used in oncology, there are also a few examples of TMA applications in non-neoplastic diseases, for example, in neuronal tissues[1], indicating that TMAs can be applied to all fields of medical research in which tissue analyses are required.

Methodology

The TMA technology allows the simultaneous analysis of up to 1,000 tissue samples on a single microscope glass slide[2,3]. Minute tissue cylinders (typically 0.6 mm in diameter) are taken from different primary tumour blocks (the 'donor' blocks) and subsequently assembled in an array-like format into one empty 'recipient' block (FIG. 2). Regular MICROTOMES can be used to cut sections from these TMA blocks. Virtually all types of *in situ* analyses applicable to traditional 'large' tissue sections

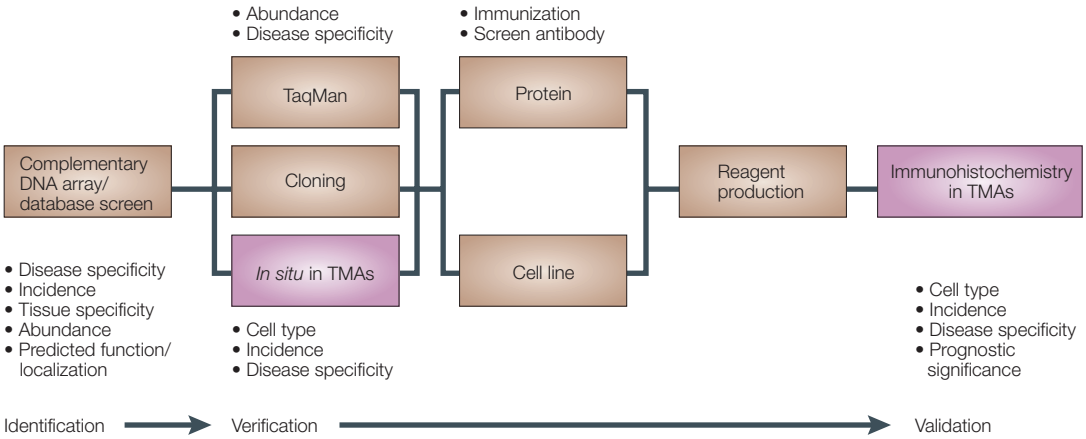

Figure 1 | **Application of the TMA technology in lead discovery and validation.** Differentially expressed target genes are identified from database screening. Expression level is confirmed by TaqMan, and the cell source and incidence determined in tissue microarrays (TMAs) by *in situ* hybridization. Selected genes are cloned and expressed, and crucial reagents, including antibodies and cell lines, generated. When antibodies are available, comprehensive protein-expression analysis is completed in TMAs.

can also be applied to TMA sections, including IMMUNOHISTOCHEMISTRY (IHC), FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) or RNA *IN SITU* HYBRIDIZATION (RNA-ISH). The small diameter of the specimen taken out of each donor block minimizes the damage to patient tissue, which might later be needed for additional diagnostic procedures, and at the same time maximizes the number of samples that can be taken out of one donor block. Paraffin-embedded tissues are generally used for TMA construction, because of the availability and the ease of handling paraffin-embedded blocks. However, frozen tissue samples can also be utilized[4,5]. In the latter case, frozen OCT Tissue-Tek compound is utilized as a carrier material instead of paraffin. Frozen TMAs are especially useful for IHC, when the antibodies are not suitable for use in formalin-fixed tissues.

TMAs and tissue heterogeneity

An early concern of using 0.6-mm TMA tissue cores for molecular analyses of cancer tissues was that the results obtained would not be representative of the entire tumour[6–8]. At least 20 studies have, therefore, compared IHC findings from TMAs and their corresponding traditional 'large' sections in various cancer types, including carcinomas of the breast[7,9–11], colon and rectum[12,13], prostate[14–16], stomach[17], kidney[18], urinary bladder[19] and brain[20]; soft-tissue tumours[21,22]; and lymphomas[23–26]. In most of these studies, several samples from each of the donor tissue blocks were included in the TMA to determine the number of punches yielding sufficiently concordant results as compared with the large-section analysis. The results of all these studies are very similar. Typically, reasonably good concordance between large-section-analysis and TMA results is found if only one punch is used, and the level of concordance increases with the use of additional punches[7,12,15,22]. No major improvement is typically found once the number of arrayed samples exceeds three or four[15].

The impact of the results of such comparative studies of large sections versus TMAs is somewhat limited, because they are based on the assumption that conventional whole sections are representative of an entire tumour (FIG. 3). However, considering the tiny volume of a typical traditional tissue section (1 × 1 cm, thickness 3 μm = 0.0003 cm^3) in comparison with the entire tumour volume, this assumption might not be true. A tumour with a diameter of 1 cm has a volume of approximately 0.52 cm^3, which is about 1,700 times more than the volume examined on a standard 'whole section' — the current 'gold standard' for molecular tissue analysis. Therefore, studies seeking for associations between molecular features and tumour phenotype or clinical outcome are more important in determining the utility of the TMA technique. In fact, all TMA studies addressing this issue fully reproduced known associations between molecular changes and clinico-pathological parameters. For example, associations were found between the expression of the oestrogen receptor and the progesterone receptor[27], and separately ERBB2 (also known as HER2/*neu*) alterations[28], and survival in breast cancer; and between KI67 LABELLING INDEX and prognosis in urinary bladder cancer[19], soft-tissue sarcoma[29] and Hurthle cell carcinoma[30].

It is notable that some data suggest that TMAs might even be superior to large sections for the detection of clinical associations. In one study, we found an association between nuclear p53 accumulation and poor prognosis of breast cancer patients when using TMA sections but not with corresponding large sections[11]. A re-analysis of the data showed that these 111 tumours — which were p53-negative on TMAs, but positive on large sections — had as good a prognosis as those tumours that were rated p53-negative on both TMAs and large sections (FIG. 4). Focal findings that were only observed on large sections had obviously no clinical significance for these patients. At least in some instances, the high degree of standardization of staining and analysis that is possible with TMAs seems to more than compensate for any possible disadvantage related to the small size of arrayed tissues.

IMMUNOHISTOCHEMISTRY (IHC). Method for the *in situ* detection of proteins in tissues using a specific antibody coupled to a chromogenic enzyme complex. The staining allows a rough estimation of the expression level and the intracellular localization of the target protein.

FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH). Method for the detection of particular DNA sequences (for example, a gene) in cell nuclei, for example, in tissue sections. A fluorescence-labelled DNA fragment complementary to the target DNA sequence is used as a probe. FISH allows the determination of the exact copy number of a target gene.

RNA *IN SITU* HYBRIDIZATION (RNA-ISH). Method for the detection of mRNA sequences in tissue sections. A (usually isotopic) labelled DNA or RNA fragment complementary to the target mRNA sequence is used as a probe. The signal intensity allows a rough estimation of the mRNA expression level.

KI67 LABELLING INDEX The Ki67 labelling index is the fraction of cell nuclei positive for staining with an antibody against the Ki67 protein. The Ki67 antigen is exclusively expressed during cell cycle.

Combining cDNA and TMAs

Complementary DNA arrays have been widely used for lead discovery. Expression profiles for tens of thousands of genes can be measured with DNA arrays in one tissue. However, cDNA array analyses are usually only done in a relatively limited number of tissue samples because of cost considerations and the limited availability of unfixed tissue samples. More than 15 studies have demonstrated the power of TMAs for the further validation of cDNA array data. Genes whose expression differs significantly between samples from normal and diseased tissues can then be profiled in hundreds of tissues in TMAs, ideally by IHC.

For example, Bubendorf *et al.*[31], studying prostate cancer, surveyed 5,184 genes for those expressed more highly in a hormone-refractory prostate cancer cell line than in its hormone-sensitive parent cell line. Insulin-like growth factor binding protein-2 (IGFBP2) was one of the most significantly upregulated genes in hormone-refractory cells. In a follow-up TMA analysis of 264 clinical prostate tissue samples, IGFBP2 protein was highly expressed in all of 30 locally recurrent hormone-refractory tumours, but only in 36% of the 204 primary tumours, and in none of 26 normal prostate specimens.

Dhanasekaran *et al.*[32] determined expression profiles of normal adjacent prostate, benign prostate hyperplasia, localized prostate cancer, and metastatic hormone-refractory prostate cancer using a cDNA microarray. The prognostic value of hepsin, a transmembrane serine protease that was strongly upregulated only in localized and metastatic prostate cancer, was confirmed by IHC on a TMA containing more than 700 clinically stratified prostate-cancer specimens.

Porkka *et al.*[33] identified overexpression of the gene encoding elongin C in prostate cancer cell lines. TMA validation experiments showed expression and high-level amplification of elongin C in eight (23%) of thirty five hormone-refractory carcinomas, but in none of thirty five untreated prostate carcinomas. Mousses *et al.*[34] validated expression of the *S100P* gene, which was found highly overexpressed in hormone-refractory xenograft tumours by cDNA expression analysis. TMA analysis of 544 clinical cancer specimens by RNA-ISH and IHC revealed that *S100P* overexpression was significantly associated with tumour progression, indicating dysregulation of this pathway in hormone-refractory and metastatic prostate cancers. In a recent study by Xin *et al.*[35], dowregulation of annexins 1, 2, 4, 7 and 11 in hormone-refractory prostate cancers, as compared with localized hormone-naive tumours, was identified by cDNA microarray analysis and subsequently confirmed on a TMA.

Similar studies have also been conducted in kidney cancer[18], gliomas[20], colorectal cancer[36], soft-tissue tumours[37], synovial sarcomas[38], thyroid cancer[39], lymphomas[40] and breast cancer[10,41,42]. If individual genes with a particular clinical relevance are detected in a cDNA array approach, TMAs are ideally suited for further validation of the data.

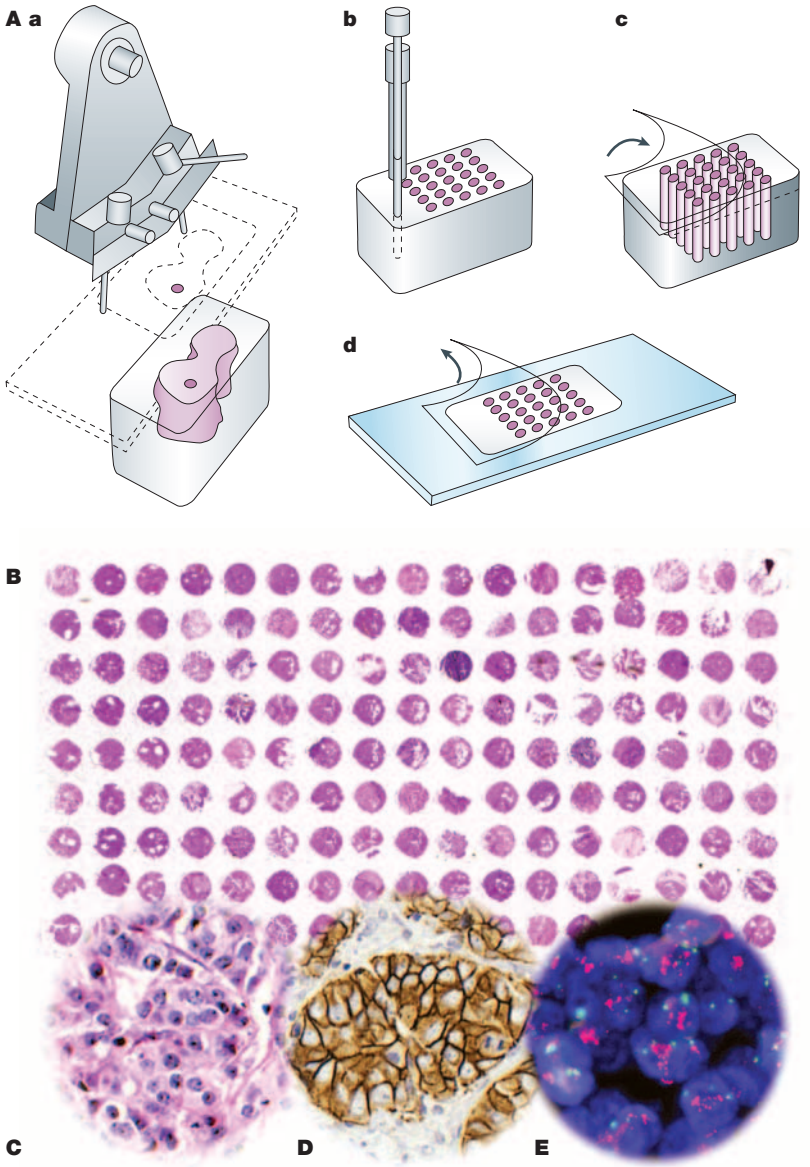

Figure 2 | **TMA manufacturing and applications. A** | Cylindric tissue cores (usually 0.6 mm in diameter) are removed from a conventional ('donor') paraffin block using a tissue microarrayer; these are released into premade holes of an empty ('recipient') paraffin block. Regular microtomes can be used to cut tissue microarray sections. The use of an adhesive-coated slide system (Instrumedics) facilitates the transfer of tissue microarray (TMA) sections on the slide and minimizes tissue loss, thereby increasing the number of sections that can be taken from each TMA block. **B** | Overview of a haematoxylin-eosin (H&E) stained TMA section. Each tissue spot measures 0.6 mm in diameter. **C**–**E** | Magnifications of sectors from tissue spots from different experiments. **C** | H&E staining of breast cancer tissue. **D** | Immunohistochemistry of breast cancer tissue using the HercepTest (DAKO). Brown membranous staining indicates strong ERBB2 expression. **E** | FISH analysis of the same case showing ERBB2 gene amplification (red signals) but normal copy numbers of centromere 17 (green signals).

Target validation and molecular epidemiology

The validation process for potential therapeutic targets includes various types of functional analyses. Molecular epidemiology is another important feature for the characterization of a new drug target. A high frequency of a molecular abnormality in a single important tumour type would be the most desirable finding. However, in practice the inverse situation is more often true. Most aberrations occur in less than 50% of samples

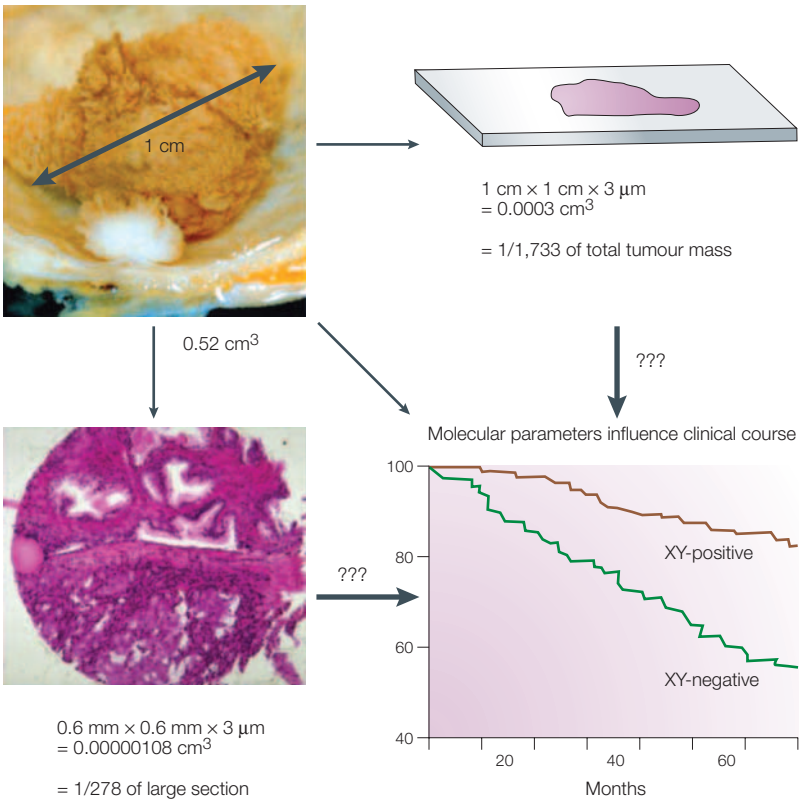

Figure 3 | **Representativity of TMAs.** Both conventional large tissue sections and tissue microarray (TMA) spots represent only a small proportion of the bulk of a tumour. A given tumour measuring 1 cm in diameter has a volume of 0.52 cm^3, whereas a conventional large section of the same tumour is about 0.0003 cm^3, and the corresponding TMA spot about 0.00000108 cm^3. Considering these numbers, the representativity problem is about sixfold larger between the large section and the whole tumour, than between the TMA spot and the large section. Rather than aiming for a comparison of results obtained from large-section and TMA analysis, molecular studies should, therefore, determine which methodology, TMA or large section, is better suited to discover molecular factors that predict the clinical course of disease.

of any individual tumour entity, but are present in many different tumour types. Theoretically, information on gene expression in different tumour entities can be collected from the literature. However, a literature search often fails to yield meaningful results. Although abundant data are available, these are usually too inconsistent to be practically useful. This is best illustrated by expression data for ERBB2, the molecular target for the monoclonal antibody trastuzumab (Herceptin; Genetech). Hundreds of studies have investigated ERBB2 amplification and overexpression in a wide variety of different tumours, but the results vary dramatically in each tumour category (TABLE 1). The most important causes of such discrepancies are methodological and assessment variations between the different studies. It is obvious that one analysis using one standardized procedure will be better suited to assess the epidemiology of a specific molecular feature than an extensive literature search. For such studies, we have generated a TMA bank consisting of more than 20,000 arrayed cancer tissues. This includes a multitumour TMA with more than 3,000 individual tumour samples from more than 120 different tumour categories. This TMA enables a rapid evaluation of therapeutic targets. For example, we found strong expression of ERBB2 in 15 different tumour categories, KIT (one of the Glivec target genes) positivity in 52, and epidermal growth factor receptor (EGFR) positivity in 71 (own unpublished data). The combination of results obtained from such a multitumour TMA with the published incidence data of major diseases allows an estimate of the total market potential of new targeted therapies (TABLE 2).

The determination of the prevalence and level of expression is not the only contribution of TMAs to the assessment of new drug targets. The analysis of the target's role in tumour progression could be equally interesting. Molecular features that play a direct part in tumour progression are often vital for individual tumour cells, and might therefore constitute better targets than upregulated genes that are not directly involved in tumour biology. TMAs are helpful for the evaluation of associations between molecular features and clinico-pathological data. 'Progression TMAs', which include tumours of all different disease stages, or 'Prognosis TMAs', which contain tumours with clinical follow-up data, are optimally suited for such studies. A multitude of studies have utilized Progression TMAs to find associations between gene amplification or protein overexpression and tumour phenotype. Examples of relevant findings include associations between cyclin E[43], fibroblast growth factor receptor-1, RAF1 (REF. 44), MDM2 or cyclin-dependent kinase-4 (REF. 45) amplification or MAGE-A4 expression[46] and stage and grade in bladder cancer; keratin-7 and keratin-20 expression and grade in colorectal carcinoma[47]; IGFBP2 expression and hormone-refractory state[31]; EIF3S3 amplification and stage[48], aneusomy and grade in prostate cancer[49]; E-cadherin expression and tumour size[50] in prostate cancer, aneusomy and tumour type in brain tumours[51]; and SHP1 expression and tumour development in lymphomas[40]. In addition, TMAs have been utilized to establish associations between shortened patient survival and expression of cyclooxygenase-2 (COX2; the molecular target of anti-inflammatory drugs such as aspirin) in breast cancer[52], expression of topoisomerase IIα (the molecular target of anthracyclins) in glioblastomas[53], expression of c-MYC and AIB1 in hepatocellular carcinoma[54], and IGFBP2 expression in prostate cancer[31]. FIGURE 5 shows the significant associations of ERBB2 and EGFR overexpression with patient survival as determined on a breast cancer TMA composed of >2,000 tumours with clinical follow-up information.

Another important but often neglected step in the ideal assessment of a therapeutic cancer target is an evaluation of the possibility of heterogeneity between findings obtained with primary tumours and those from their metastases. Although metastases are typically the target for therapy, it is almost always the primary tumour that is assessed for molecular alterations, both for convenience and because it is generally believed that primary tumours and metastases are genetically similar. Only a few studies have compared molecular features in large numbers of primary tumours with those in metastases. Such projects are difficult to undertake,

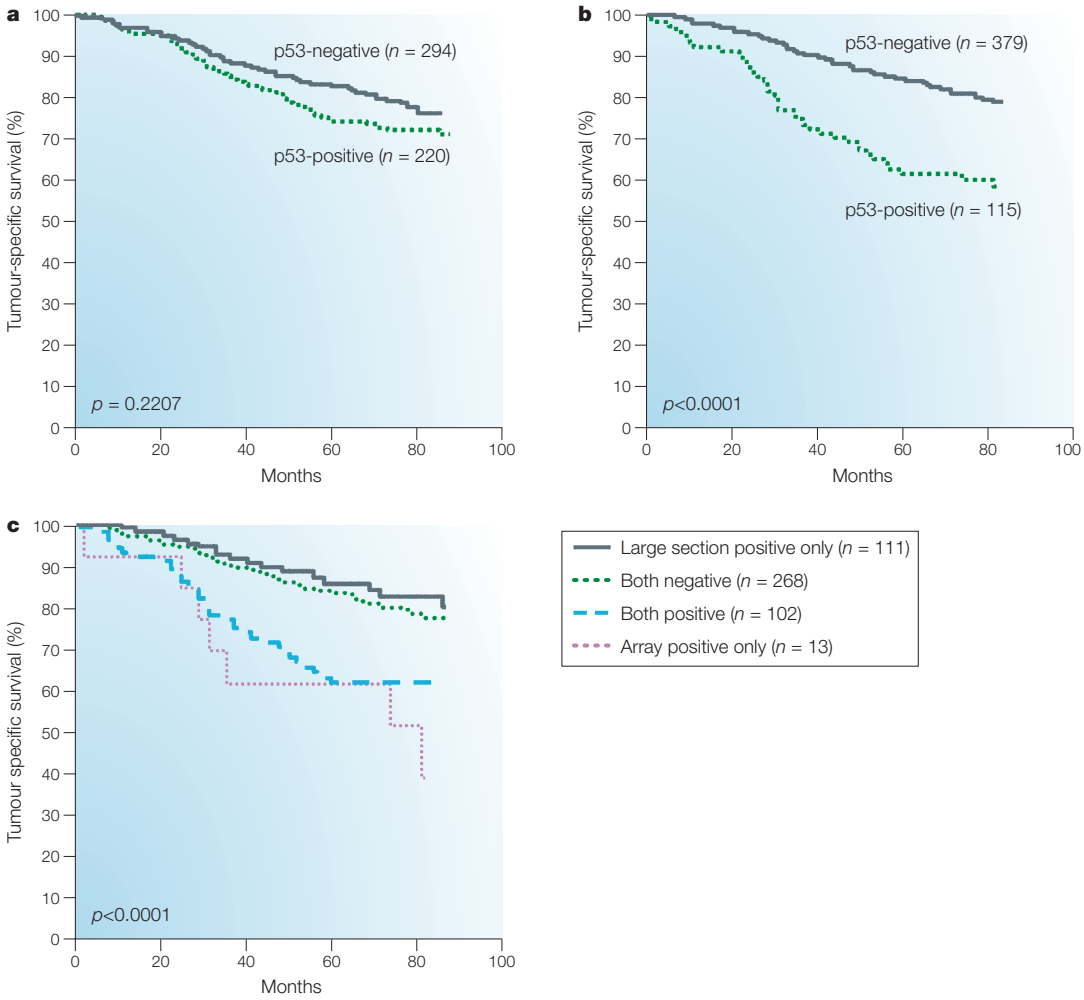

Figure 4 | **Comparison of p53 data obtained from TMAs and whole sections.** Kaplan–Meier plots showing the relationship between p53 immunohistochemistry results and patient survival in large section (**a**) and tissue microarray (TMA) analysis (**b**). **c** | Combined analysis (for explanation see text).

because of the large amount of tissue needed for a meaningful analysis and the limited availability of metastatic tissue material. TMAs composed of sets of paired primary tumours and nodal or distant metastases are important tools to rule out significant heterogeneity between primary tumours and metastases. In one such study we utilized a metastasis TMA to demonstrate a good concordance of ERBB2 status between 196 primary and metastatic breast cancers[55].

Normal tissue cross-reactivity

Assessment of the expression of drug targets in vital tissues is an important component of drug development. For example, the US FDA requires a survey of expression in 32 different organs as part of tissue cross-reactivity studies. Several established drug targets, such as CD20 (the target for rituximab (Rituxan; Genetech/IDEC)) and EGFR, are expressed in normal cells. Consistent with this finding, the treatment of patients with rituximab results in the depletion of both malignant and non-malignant B cells[56]. Exact knowledge of where therapeutic target genes are overexpressed in normal tissues might allow the prediction of side effects of new therapies. In contrast to multitissue NORTHERN BLOTS or protein arrays, TMAs have the advantage that individual cell types can be assessed for target gene expression. Because of the variety of different cell types residing in one organ, simple expression analysis on disintegrated tissue — as, for example, obtained by Northern blot — might not provide optimal information in many instances.

Assay development

The development of an assay that reproducibly identifies patients that would benefit from a new drug is often an important prerequisite for a successful targeted anticancer therapy. For example, VYSIS' PathVysion FISH test or DAKO's HercepTest are FDA-approved assays for identifying patients that could benefit from treatment with trastuzumab. For assay development, the strength of the TMA approach lies in the small size of individual tissue samples. If consecutive sections

NORTHERN BLOT
Method for the detection of mRNA sequences in isolated total cellular RNA that has been separated according to the mRNA length using an isotopic labelled antisense DNA or RNA probe. The signal intensity allows a rough estimation of the mRNA expression level, whereas the signal localizations enables to estimated the target mRNA size.

Table 1 | **Frequency of ERBB2 expression***

Tumour type	Expression (%)	Amplification (%)	References
Breast carcinoma	13–100	6–55	9,62–72
Ovarian carcinoma	9–89	0–66	62,73–83
Colon carcinoma	0–83	2–50	62,73,84–88
Endometrial carcinoma	17–88	0–63	62,89–95
Gastric carcinoma	8–56	6–18	62,96–103
Head and neck cancers	0–93	0–43	62,104–112
NSCLC	4–100	0–18	62,73,113–122
Prostate carcinoma	0–100	0–53	62,123–133
Urinary bladder carcinoma	2–74	3–26	62,133–140

*Determined by immunohistochemistry or reverse transcriptase-polymerase chain reaction and ERBB2 amplification in different tumour types as determined by fluorescent *in situ* hybridization or Southern hybridization. NSCLC, non-small-cell lung cancer.

are used from one TMA, all comparisons will be carried out on tissues that are virtually identical, both genetically and with respect to tissue processing. In one study we used TMAs to investigate various assays for EGFR determination using a number of different antibodies and FISH. The results for some of the more important tumour types are summarized in TABLE 2. These results illustrate the difficulty of quantifying EGFR expression by IHC and highlight the potential influence of an assay's characteristics on the assessment of target expression for a new anticancer drug. For example, if a FISH analysis for gene amplification was predictive of response to anti-EGFR therapies, the number of new patients that might eligible for therapy in the United States and in Europe would be around 10,000. However, if EGFR expression using Zymed's anti-EGFR antibody were predictive, the target patient population might be more than 800,000.

Inclusion of controls into the original TMA design, such as cell lines (as provided with DAKO's HercepTest), synthetic materials (protein, RNA or DNA) or small, normal-tissue control sections, facilitates the comparison of TMA experiments carried out at different times, as well as by different laboratories.

Clinical studies

The example of EGFR expression, its measurement and the response to anti-EGFR therapies shows how difficult the prediction of response to targeted anticancer therapy can be. Although individual tumours have responded well to anti-EGFR therapy, no association was found between EGFR expression, as determined by IHC, and benefit from treatment in any of these studies. It is probable that biological features other than the absolute level of EGFR expression determine whether or not a tumour responds to such a therapy. For example, the functional status of downstream genes in the EGFR pathway could have a relevant influence on the reaction of a cell to EGFR blockade. To identify such 'hidden' predictive biomarkers, access to tissues from patients treated with a new drug and to the corresponding outcome information is of crucial importance. Therefore, tissues from patients included in clinical trials are a key resource for the identification of predictive biomarkers. TMAs are highly useful for the economical use of these precious tissues. Dozens of genes associated with the EGFR pathway can be rapidly analyzed and their influence on response to therapy can be assessed on such TMAs.

Table 2 | **Influence of EGFR detection methods on the number of patients that might benefit from anti-EGFR treatment**

Tumour type		EGFR-positive samples (%)				Patients	Patients for anti-EGFR therapy			
	n	FISH	IHC 1 (Zym.)	IHC 2 (Novo.)	IHC 3 (Chemi.)	(EU+US)	FISH	Zym.	Novo.	Chemi.
Lung (squamous cell carcinoma)	44	0	65.2	15.2	4.4	169,000	0	110,118	25,688	7,436
Lung (adenocarcinoma)	41	2.4	39.6	6.5	4.3	105,960	2,543	41,960	6,887	4,556
Lung (small-cell carcinoma)	19	0	2.3	0	2.3	50,770	0	1,168	0	1,168
Breast (ductal cancer)	42	0	4.3	19.5	7.3	312,985	0	13,458	61,032	22,848
Breast (lobular cancer)	37	0	0	0	13.5	47,230	0	0	0	6,376
Ovary (serous carcinoma)	41	0	13.3	6.8	2.2	30,27	0	4,027	2,059	666
Oesophagus (squamous-cell carcinoma)	29	3.4	85.3	70.6	3	32,720	1,112	27,910	23,100	982
Colon (adenocarcinoma)	41	0	4.4	9.5	2.4	262,740	0	11,561	24,960	6,306
Hepatocellular carcinoma	59	0	1.3	8.4	0	36,390	0	473	3,057	0
Pancreas (adenocarcinoma)	35	0	40.9	24.5	0	53,530	0	21,894	13,115	0
Kidney (clear-cell carcinoma)	34	2.9	61.6	4.2	4.3	42,580	1,235	26,229	1,788	1,831
Urinary bladder (TCC invasive)	39	2.6	42.9	32.4	10.3	46,080	1,198	19,768	14,930	4,746
Prostate carcinoma (untreated)	44	0	9.1	8.7	2.2	257,160	0	23,402	22,373	5,658
Glioblastoma multiforme	39	48.7	75	46.5	40.9	1,664	810	1,248	774	681
Malignant fibrous histiocytoma	25	8	44	0	0	2,620	210	1,153	0	0
All types						3,774,313	10,107	140,006	395,729	834,336

The numbers of patients for anti-EGFR therapy do not add up to the numbers given in the last row because not all EGFR-positive tumour types are displayed in the table. EGFR, epidermal growth factor; FISH, fluorescence *in situ* hybridization; Zym., Zymed Laboratories, Inc.; Novo., Novocastra Laboratories Ltd; Chemi., Chemicon Intl.

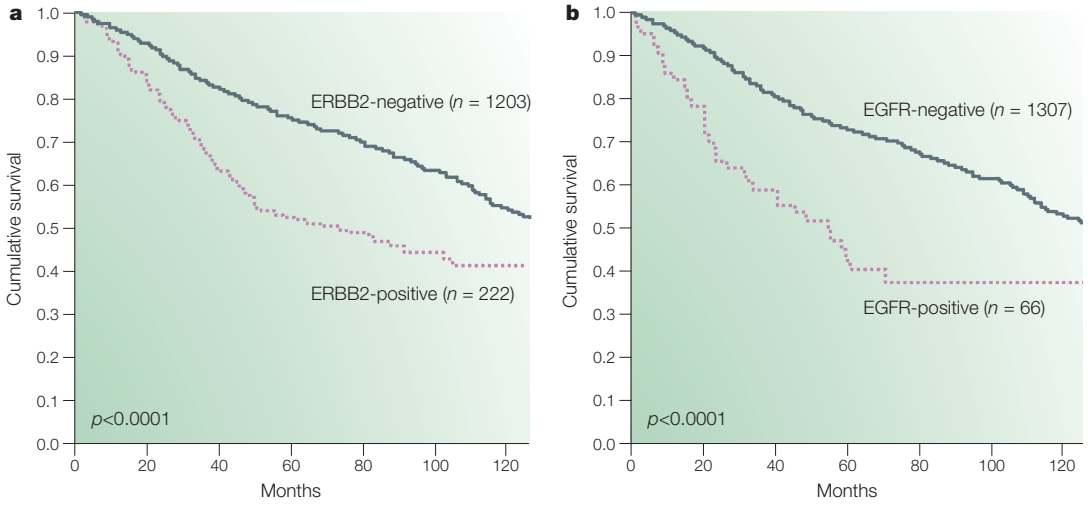

Figure 5 | **Associations of gene overexpression with patient survival as determined on a breast cancer TMA composed of >2,000 tumours with clinical follow-up information. a** | ERBB expression data. **b** | EGFR expression data. These data are examples of the power of the tissue microarray (TMA) method for the almost immediate clarification of the prognostic significance of molecular tumour alterations. EGFR, epidermal growth factor receptor.

Automation

The analysis of many genes or gene families in many tissues pushes the limits of 'manual' analysis. Even though a trained pathologist can interpret 500 or more TMA samples per hour, this rate is insufficient for large-scale studies and, moreover, cannot be continued for an unlimited period of time. One technician could easily immunostain more than 200,000 spots per week; Genentech's TMA database already contains more than 7 million data points. Automated analysis, appropriate database solutions and advanced statistical analyses are crucial for generating and understanding the results.

In principle, TMAs are highly suited for automated analysis. The greatest difficulty for automated tissue analysis — the selection of an appropriate tissue area — has been solved to a large extent during TMA manufacturing. Several sophisticated trend-setting systems enabling automated TMA analysis have recently been introduced. Some of these utilize fluorescent dyes for immunostaining because of the greater dynamic range as compared with peroxidase-based systems and the potential for multicolour analyses. For example, Camp *et al.* described an automated quantitative analysis system using several fluorescence tags that was able to separate tumour from stroma cells and to define subcellular compartments[58]. Others have used simultaneous double-direct immunofluorescence detection of one test and one reference antigen in order to normalize for the cellular content of detectable protein in each TMA spot[59].

The most significant limitation of fluorescence-based immunostaining is the need to develop complex staining protocols for multicolour applications and the difficult manual re-evaluation of questionable staining results. It must also be remembered that highly sensitive labels do not solve all of the inherent problems of IHC, which are mostly caused by non-homogeneous tissue processing and fixation. Most commercial products for automated TMA analysis, therefore, concentrate on conventionally immunostained TMA sections. These are generally based on an automated microscope with a charged-coupled device (CCD) camera. An overview image is generated that is used to identify the localization of each tissue spot in the TMA for subsequent high-resolution scanning. Several systems allow the user to create a virtual slide that supports navigation and different magnifications, just as with a real microscopic slide. Alternatively, scanner technology can be used instead of a CCD camera. For example, Aperio's ScanScope system can acquire a typical 20 × 30 mm TMA area in less than 20 minutes. Such 'low tech' solutions for quantifying the total signal intensity per TMA spot are easy to establish and surprisingly efficient for the identification of clinico-pathological associations. For example, the analysis of several biomarkers with our custom-made system revealed the expected associations with survival time in all cases. As an example, the results obtained for manual and automated oestrogen receptor staining analysis in breast cancer are shown in FIG. 6 (M. Ramseier, S. Hänggi, J. Wirth, personal communication).

For isotopic *in situ* hybridization, quantification of signal on a PHOSPHORIMAGER correlates highly with other quantitative techniques, such as Taqman, and can be completed within 24 hours of hybridization, as opposed to the usual two to four weeks. Measurement is objective and, similar to immunofluorescence, has a greater dynamic range than traditional semi-quantitative evaluation by eye.

TMA informatics: data management

The analysis and management of data generated from TMA experiments is significantly more challenging than the process of building and sectioning the array.

PHOSPHORIMAGER
Device for visualization of radioactive signals originating from experiments such as isotopic RNA-ISH or Northern blots, comparable with a radiosensitive photographic plate.

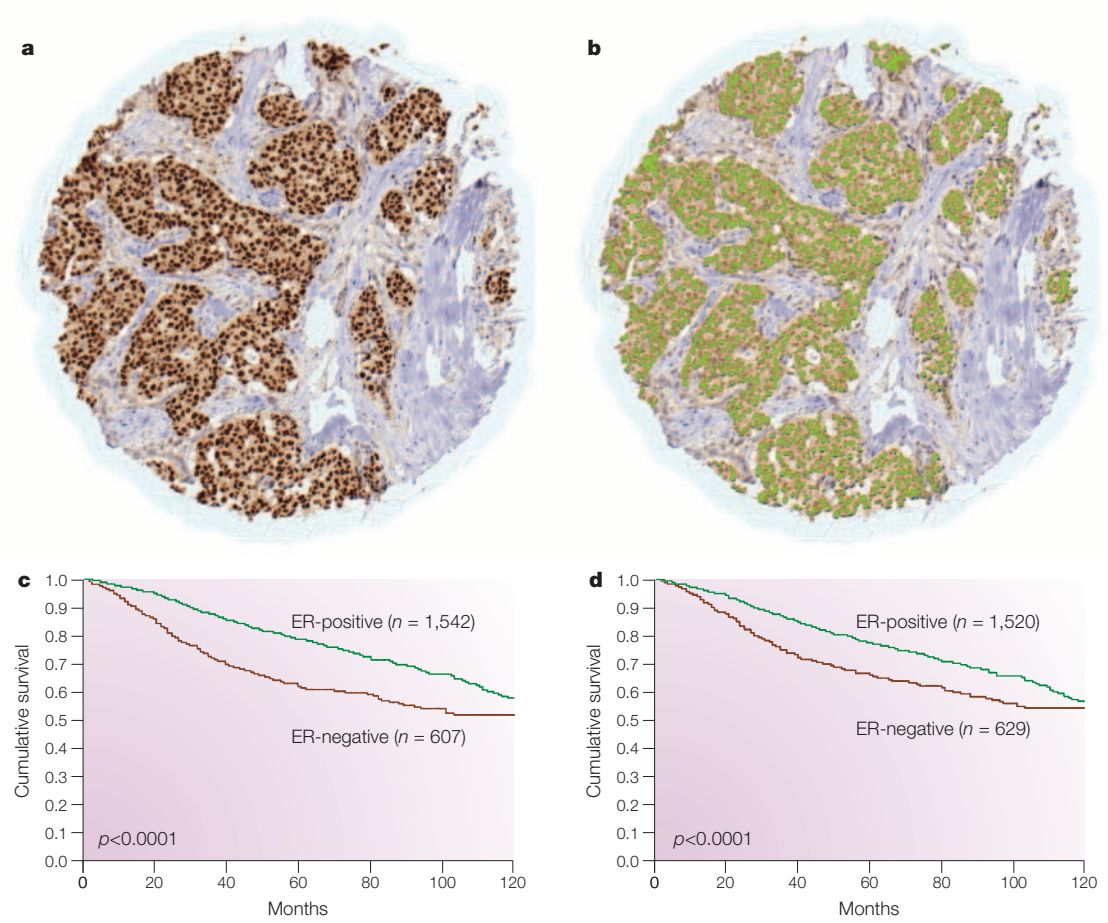

Figure 6 | **Automated versus manual TMA analysis. a** | Immunohistochemistry showing oestrogen receptor (ER) expression (brown nuclear staining) in a breast cancer tissue spot. **b** | Same image as in **a** with green colour indicating automated thresholding for positive ER staining. Survival plots calculated from both manual (**c**) and automated (**d**) analysis confirm the worse prognosis for patients with loss of ER expression.

Many laboratories have developed data-management tools of increasing sophistication using spreadsheet or database software such as Microsoft's Excel or Access. Although these programs are suitable for relatively small sets of data, they are unlikely to provide scaleable long-term solutions. Fully relational databases capable of handling much larger data sets have been built and are likely to be the way of the future. Most programs include graphical maps of core locations in the array, both for assistance in design and for entering results from slides.

Captured images can be stored with, or linked to, experimental and core information in the database. Many systems are able to display the images on a web-browser for subsequent review and data entry by pathologists. Virtual retrieval of stored images also allows any scoring disparities between investigators to be resolved without the need to physically exchange slides between multiple sites. Several software programs for TMA image storage and retrieval are available in the public domain (see Further information). Image storage and retrieval increases the data storage and handling demands on a TMA information-management system exponentially.

As numeric and image data volume grows, customized reporting tools are increasingly essential for efficient data retrieval and analysis. The Stanford group has recently published and made available tools that greatly assist the analysis of large TMA datasets[60]. The TMA-Deconvoluter is able to reformat the semi-quantitative scores conventionally used by pathologists into numeric data files that can be used for statistical analysis and/or to generate hierarchical clusters using the Cluster and TreeView software packages.

Standards for TMA data exchange

Commonly accepted minimal standards for sharing TMA core source information are thought to facilitate the sharing of TMAs and data produced in different institutions. Discussion among investigators in the United States has recently resulted in the creation of a preliminary XML data-exchange working standard[61]. Here it is most important that the details of experimental conditions, assay instrumentation, scoring criteria and other experimental variables are considered if results obtained in different institution are either combined or compared with each other.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

Online links

DATABASES

The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
AIB1 | c-MYC | COX2 | cyclin-dependent kinase-4 | cyclin E | E-cadherin | EGFR | EIF3S3 | elongin C | ERBB2 | fibroblast growth factor receptor-1 | hepsin | IGFBP2 | keratin-7 | keratin-20 | MAGE-A4 | MDM2 | oestrogen receptor | progesterone receptor | RAF1 | *S100P* | SHP1 | topoisomerase IIα

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National Human Genome Research Insitute's Tissue Microarray Project:
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http://www.nhgri.nih.gov/DIR/CGB/TMA/construction.html

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